MECHANISM OF PIGMENT DISPLACEMENT IN UNICELLULAR CHROMATOPHORES

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Introduction

Several hypotheses have been proposed regarding the basic mechanism which determines the flow of pigment granules back and forth in the protoplasmic branches of unicellular chromatophores. The melanophores of vertebrates, for example, have been regarded as amoeboid cells with pseudopodia which extend and retract alternately (Hooker, 1914); and as modified visceral muscle cells (Spaeth, 1916) More recently, Shanes and Nigrelli (1941) have related the movement of the melanin granules to processes occurring in some birefringent material which appears to lie between and around the melanophores proper.

The present investigation deals with the action of hydrostatic pressure on the melanophores of the isolated scale of *Fundulus heteroclitus,* and the work forms a sequence with several previous studies. Amoeboid movement (Brown and Marsland, 1936), the cleavage movements of various marine eggs (Marsland, 1938 and 1939a), and protoplasmic streaming in plant cells (Marsland, 1939b) were all found to be activated by intracellular processes of gelation and solation (Marsland, 1942). Protoplasmic gelations have proved to be uniquely susceptible to the influence of hydrostatic pressure (Marsland and Brown, 1942), and consequently an opportunity is afforded for studying the role of such reactions in the behavior of the melanophore.

Experimental Results

Effects on pulsating melanophores

Pulsation of the melanophores of the isolated scale was induced by a modification[1] of the method of Spaeth (1916), and the scale was then placed in the microscope-pressure chamber (Marsland and Brown, 1936). This chamber permits the melanophores to be viewed at a magnification of 600 diameters at pressures ranging up to 8000 lbs./in.^{2}.

At 1000 lbs./in.^{2} the pressure effect upon the pulsating cells is scarcely discernible; but at 2000 lbs. the change becomes very obvious. The flow of the pigment granules back and forth in the branches of the melanophores continues, but the amplitude of each pulsation is plainly reduced. This reduction is confined, how-

[1] The scales were scraped from the dorso-lateral surface of the fish and placed in N/10 normal NaCl solution for 15 mins. before transfering to N/10 BaCl for seven minutes. After this the scales were returned to fresh NaCl solution in which the pulsations would start in about one hour and continue for more than two hours. A thorough two-fold washing of the scales in separate samples of each solution was done before they were introduced into the pressure chamber.

ever, to the central ends of the branches of the melanophores. The outflow of pigment granules continues to reach the distal ends of all branches, but the inflow reverses itself before the granules have fully reached the central protoplasmic mass. Thus at the maximum of each concentration the melanophore displays a number of short stumplike excrescences which correspond to the number of branches which are fully developed at maximum expansion.

At pressures between 3000 and 6000 lbs./in.^{2}, the amplitude of the pulsations is reduced more and more, entirely at the central end. The outflow still reaches the tips of the branches, but the inflow, at the higher pressures, is so restricted that almost the full length of each branch remains when the concentration of pigment is complete. Finally, at pressures between 7000 and 8000 lbs./in.^{2}, the pulsations cease altogether. Now all the melanophores remain in their completely expanded form.

If the pressure is suddenly released, an immediate concentration phase always sets in. This "release contraction" is very rapid, and it endures somewhat longer than the "contraction" which occurs in the ordinary rhythm of an uncompressed specimen.

Effects on contracted[2] *melanophores*

Since pressure inhibits and finally abolishes the contraction phase of the pulsation cycle, it was of interest to determine how the melanophores might react in the presence of reagents which induce the pigment to remain in the concentrated state. Most of these experiments were done with scales immersed in N/10 KCl solutions, although the same results were also obtained with adrenalin (1:1000). The melanophores of uncompressed control scales in these solutions remained in a fully contracted state for a period far in excess of the time required for the experiments.

The results of these experiments are indicated in figure 1. The several photographs are of the same melanophore successively exposed to different degrees of pressure. The response to increased pressure is always an immediate expansion, and the degree of this expansion bears a direct relation to the intensity of the pressure up to 7000–8000 lbs./in.^{2}. At this level a maximum dispersal of the pigment is always observed.

The degree of expansion depends directly upon the intensity of the pressure. The same configuration is reached at a certain pressure regardless of whether the melanophore expands to this point, as a result of increasing the pressure, or contracts as a result of decompressing. Also when a fixed pressure is maintained, the characteristic degree of expansion is maintained for many minutes. But whenever the pressure is completely released, the melanophores return immediately to a fully contracted state.

Experiments with denervated melanophores

Since Parker (1934) has shown that the terminal branches of the nerves which supply the melanophores retain some influence on the contractile state even after

[2] The term *contraction* will be used to designate the process whereby the pigment undergoes concentration in the melanophore, despite the fact that this process is not entirely comparable to the contraction of a muscle. This usage seems justifiable in view of the fact that pigment concentration probably does involve a contraction of the plasmagel system of the chromatophore, although the cell as a whole does not contract (see p. 259).

all connections with the central nervous system have been severed, it was necessary to determine whether or not the action of pressure is mediated through the activity of the surviving nerve remnants. Therefore the foregoing experiments were repeated, using melanophores in which complete denervation was assured.

For this purpose the method of Parker was used. Broad dark bands were established in the caudal fins of ten medium sized Fundulus, by making dorso-ventral cuts completely through the fins about two mm. caudal to the origin. After ten days the bands were not quite as wide as originally, but they were still clearly discernible as darker areas in light-adapted fish and as lighter areas in dark-adapted specimens. In accordance with Parker's conclusion, these observations indicate that the degeneration of the residual nerves is complete within ten days, and that longer periods must elapse before secondary innervation can occur.

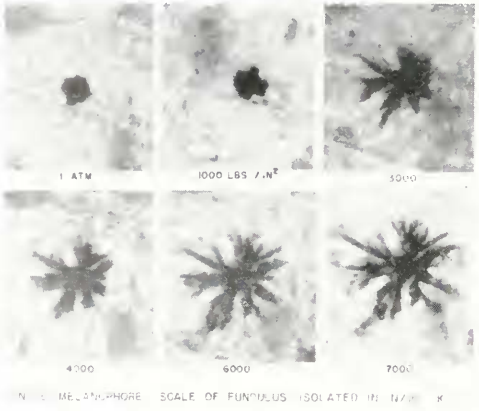

FIGURE 1. Progressive inhibition of contraction by hydrostatic pressure (courtesy of Iowa State College Press, Ames, Iowa).

On the eleventh day a small square section of the tail-fin, so selected that about half the area was derived from the denervated region, was excised, washed in three changes of N/10 KCl, and placed in the pressure chamber, which also contained the same solution. All the melanophores of the excised piece were fully contracted and there was no discernible difference between the melanophores of the innervated and denervated areas. This proves that the contracting action of the KCl solution does not depend upon the survival of the terminal nerves.

Different pressures, ranging up to 8000 lbs. in., gave exactly the same results as were described previously. Both groups of melanophores, regardless of the presence or absence of surviving nerves, gave the same degree of expansion with each increment of pressure, and in both groups a full expansion was reached at 7000–8000 lbs. This proves that the pressure acts directly upon the melanophores per se, rather than indirectly, via surviving nerve elements.

Centrifuging experiments

The foregoing experiments indicate that pressure exerts an inhibiting effect upon some protoplasmic reaction which determines the contractile state of the melano-

phore. Apparently the equilibrium of this reaction can be shifted by each increase or decrease of pressure in the range up to 8000 lbs./in.^{2}. In this respect the activity of melanophores closely resembles amoeboid movement, cleavage and cyclosis. And since these physiological activities are known to be determined by sol-gel changes occurring in the protoplasm, centrifuging experiments were undertaken, on the hypothesis that similar factors are involved in the present case.

It soon became apparent that melanophores are very unfavorable for measuring gelational changes in the protoplasm. Very high centrifugal forces must be employed before any sign of pigment displacement can be obtained and, due to the highly irregular form of the melanophore, quantitative measurements of the degree of displacement are quite impossible. However, certain qualitative indications were obtained when the isolated scales were subjected to a centrifugal force of 70,000 gravity in an air turbine ultracentrifuge.[3]

In the centrifuging experiments it was necessary to find a method for holding the centrifuged scale in a position such that the anterior-posterior axis of the scale (and consequently the plane occupied by each melanophore and its processes) was parallel to the centrifugal axis. This was accomplished by rolling the scale into the form of a cylinder and inserting it into a short length of pyrex capillary tubing, sealed at the centrifugal end. The diameter of the lumen of the tubing was about half the width of the scale. Consequently when the elastic scale begins to unroll, its outer surface becomes firmly pressed against the inner surface of the tubing. The external diameter of the pyrex tubule was only slightly less than the internal diameter of the metal jacket in the head of the ultracentrifuge, and consequently the tubule axis and the axis of the centrifuge were approximately identical. Both the metal jacket and the pyrex tubule were filled with the immersion solution, and this arrangement tended to reduce the force of the impingement of the glass tubule upon the bottom of the metal jacket.

Assuming that the resistance to the displacement of the melanin granules through the protoplasm of the melanophore provides an index of gelation, the experiments support the view that the protoplasm is set more firmly in the contracted than in the expanded melanophore. Using a standard force of 70,000 gravity and a fixed period of three minutes, no displacement of pigment was ever observed for contracted melanophores.[4] But an easily discernible pigment displacement (see figure 2) was always obtained under the same conditions with expanded specimens. Moreover, essentially the same results were obtained regardless of the agency used to induce contraction (KCl and adrenalin solutions), or expansion (NaCl, acetyl choline,[5] and physostigmine[6] solutions).

Watching the melanin granules redistribute themselves after they have been displaced centrifugally, also provides an indication that the protoplasm is in a relative state of sol when the melanophores are expanded. A complete redistribution of the granules, after they are displaced as in figure 2, appears to depend upon Brownian movement. At any rate an exceedingly active Brownian movement can clearly

[3] Cordial thanks are extended to Dr. E. Newton Harvey and to Dr. Ethel Brown Harvey for their kindness in permitting the use of this equipment at the Marine Biological Laboratory.

[4] In contracted melanophores no sign of pigment displacement was obtained with even greater forces (up to 125,000 gravity) employed for periods up to 12 mins.

[5] Acetyl choline chloride, Merck, 1×10^{-4}, in N/10 NaCl.

[6] Physostigmine sulfate, Merck, 3×10^{-6}, in N/10 NaCl.

be seen as the melanin granules begin to invade the part of the protoplasm which previously was cleared of the pigment. This movement continues for about an hour, at which time the redistribution is almost complete.

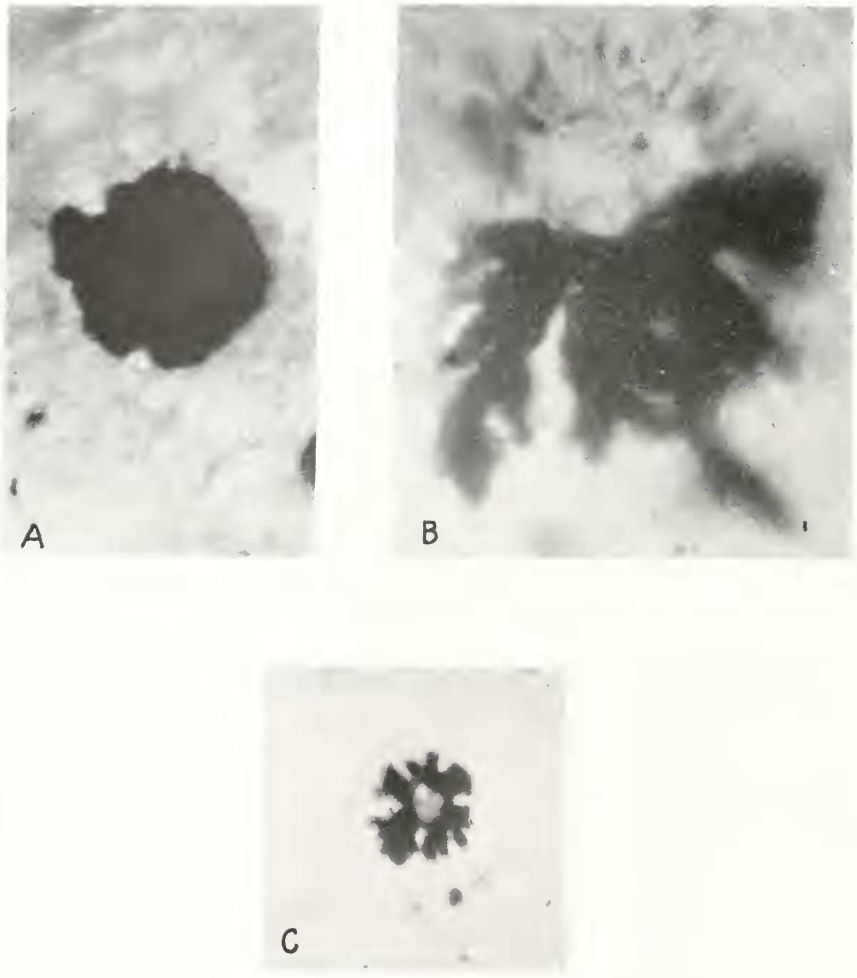

FIGURE 2. (*A*) contracted, and (*B*) expanded melanophores, both centrifuged for 6 minutes at a force of 70,000 gravity. No pigment displacement was ever obtained in contracted specimens, using forces up to 125,000 gravity. (*C*) semi-expanded specimen, showing the central hyaline plasmasol region.

Despite the drastic centrifugal treatment, the melanophores do not appear to be damaged. After the melanin granules have been redistributed and the normal expanded form has been regained, the melanophores are susceptible to further contractions and expansions, if the scales are successively immersed in solutions of KCl and NaCl.

Temperature experiments

Since it is known that the magnitude and even the sign of the pressure effect upon certain physiological processes (bioluminescence, Brown, Johnson and Marsland, 1942; and muscular contraction, Brown, 1934) depend upon temperature, some of the experiments were repeated at low (6° C.) and high (30° C.) temperatures.

These latter experiments demonstrate that high temperature fosters a contraction of the melanophores; whereas low temperature tends to induce expansion. At room temperature (20–22° C.), melanophores immersed in N/10 NaCl are all expanded; but at 30° C. they all reach a full state of contraction. Moreover, at room temperature melanophores in N/10 KCl are fully constricted; but at 6° C., a majority on a given scale are about two-thirds expanded, and the others are fully expanded. Also at 30°, when fully contracted KCl-immersed specimens are subjected to hydrostatic compression, it is very difficult to initiate expansion. In fact, the first sign of this effect does not appear until the pressure reaches about 6000/in.^{2}, in contrast to the room temperature level, namely 1000–2000 lbs./in.^{2}. At 30° C., however, the viability of the melanophores is limited to about two hrs., at least in solutions containing only NaCl or KCl. After this time irritability is lost rapidly, and no further contractions or expansions can be elicited.

Discussion

The action of pressure is localized in the melanophore itself, rather than in the surviving nerve supply, as is clearly established by the work on denervated specimens. Thus it seems likely that pressure has its main effect upon some intrinsic component in the protoplasmic system.

Recent studies on the biological effects of pressure indicate that pressure exerts at least two main types of action in protoplasmic systems. The first type of action appears to be primarily chemical, in that the pressure modifies the velocity or equilibrium of one or more of the metabolic reactions which energize the physiological process; but the second effect is physical, in that pressure appears to change the viscous and tensile properties of specific gel structures in the cell.

No doubt both types of pressure effects are present in any given system, but the first kind of action seems to dominate in the studies on muscle, nerve, and luminescence; whereas the second is dominant in amoeboid movement, cleavage, and cyclosis (Marsland, 1942).

The present evidence indicates that the action of pressure upon melanophores is mainly of the second type, and that sol-gel changes are definitely concerned with the development of the forces which cause the granules to flow back and forth in the protoplasmic branches of the pigment cells. However, quantitative measurements to substantiate this view could not be obtained, and consequently the qualitative evidence must be considered very carefully.

Pressure determines the dispersion and concentration of the melanophore pigment in a very regular and decisive fashion, and this action is clearly parallel to the pressure effects upon amoeboid movement, cleavage, and cyclosis. This leads to the conclusion that sol-gel changes are likewise of critical importance in melanophore activity. According to this view, contraction of the melanophore is determined by a gelation of the protoplasm; whereas expansion depends upon solation.

This hypothesis permits a logical interpretation of the observed effects of both temperature and pressure. The gel system of the melanophore clearly conforms to that of the several other protoplasmic gels which have been studied, in that it behaves like a type III gel (Freundlich, 1937). This type of gel *sets more firmly with increasing temperature* and, in setting, undergoes a small but *definite increase of volume*. Type III gels invariably undergo solation when the pressure is increased, or when the temperature is lowered (Marsland and Brown, 1942). Consequently the facts: that both high pressure and low temperature regularly cause expansion in the melanophore; and conversely, that decreasing pressure and increasing temperature regularly bring about contraction, may be considered as strong evidence in favor of the hypothesis.

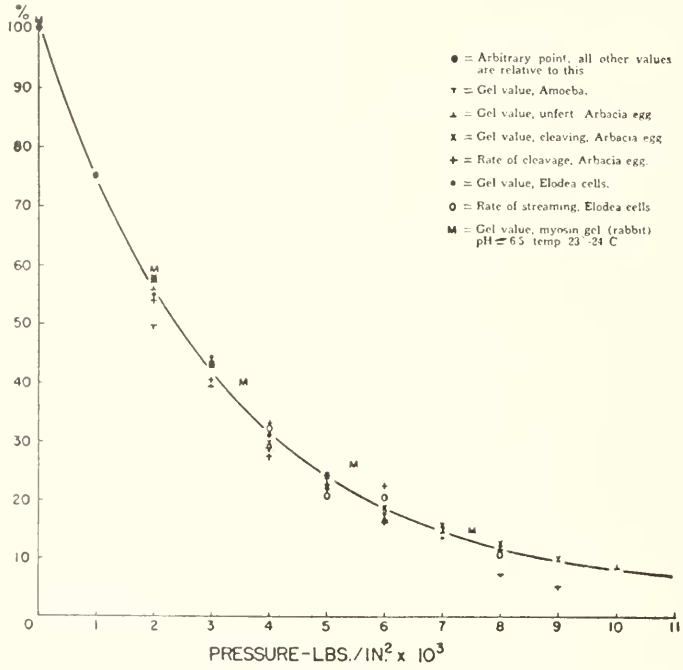

FIGURE 3. Inhibition of protoplasmic movements in relation to the solating effects of hydrostatic pressure.

A substantiation of this view must await more data, but the existing evidence seems very significant. The contraction phase of the pigmentary response is limited by pressure in a manner that parallels, at least roughly, the inhibition of gelation which has been demonstrated in plasmagel systems generally. The "half-expanded" state of the chromatophore (Fig. 1) occurs at about 2500 lbs./in.² (at 20°–22° C.), which corresponds to a gelation value of approximately 50 per cent (Fig. 3); and it seems probable that the other values, should they become available, will likewise fall upon the general curve. Also the centrifuging experiments provide at least a qualitative demonstration that gelation and solation are of critical significance in melanophore activity. Regardless of the agent (NaCl, physostigmine and acetyl-

choline solutions) which was used to bring about the pigment dispersal, expanded melanophores always showed a clear displacement of the melanin granules at a centrifugal force of 70,000×g.; whereas this force never gave any sign of pigment displacement in contracted melanophores.

It seems worthwhile to speculate briefly as to how gelation may instrument the movement of the pigment from branches into the body of the chromatophore. The simplest concept of the mechanism comes from the views of Lewis, 1942, and Marsland, 1942, which were derived from studies on amoeboid movement and cleavage. According to this view every gel, by virtue of its intrinsic structure, tends to contract spontaneously. The gel is conceived of as a colloidal network of interconnected protein units, the interstices of which are filled with a fluid residuum of the original sol. Conditions which foster gelation lead to a strengthening of the interconnecting bonds and to a folding of the extended protein units which constitute the framework of the gel. These factors account for the contractile tendency, which is accompanied by an exudation of sol, expressed from the shrinking interstitial spaces.

In the melanophore, the pigment granules are probably enmeshed in, or attached to, the gel framework which, in the expanded specimen, extends out into the outlying branches. During contraction, conditions favoring a firmer setting of the gel are imposed upon the system and consequently the gel framework begins to shrink and pull the pigment granules inward from the extended branches. Simultaneously, however, these must be an outflow of sol into the branches, in sufficient quantity to compensate for the volume of the retreating gel and pigment.

Direct observations of the melanophores under oil immersion tends to support the foregoing hypothesis. In the partially and fully expanded specimen the central region of the body of the melanophore is clearly hyaline, and the pigment granules are located only in the peripheral parts of the body and in the extended branches (Fig. 2). This differentiation indicates that the plasmasol and plasmagel of the melanophore occupy the same relative positions in amoeboid cells generally. Consequently it is proposed that the hyaline protoplasm of the body of the melanophore be considered as the *plasmasol,* which is surrounded by the pigmented outlying protoplasm, the *plasmagel.*

During contraction the central hyaline plasmasol region of the melanophore becomes obliterated. No doubt this results from the encroachment of pigment granules which come in from the branches. But in the process of this obliteration, probably, the framework of the plasmagel must shrink, exerting a pressure upon the enclosed sol. Thus part at least of the sol must seep out through the meshes of the surrounding gel and escape into the branches to replace the material which is retreating from these parts. Such an exudation of hyaline sol through the meshwork of the surrounding gel would be homologous to the escape of sol which occurs through the plasmagel sheet at the advancing tip of a pseudopodium in Amoeba (Mast, 1926).

The outlying branches of the melanophore persist during contraction (Matthews, 1931). But no one has been able to observe the outflow of hyaline sol, which must inevitably occur while the pigment is retreating from the branches. The difficulties of such observations are, no doubt, first that the sol is completely hyaline, and second, that the branches tend to be obscured by other tissues occupying a more superficial position on the scale (Matthews, 1931).

Indirect evidence of the outflow of sol may be obtained by observing (oil immersion) the inflow of pigment granules from the peripheral tips of the branches of the melanophore during the early stages of contraction. These granules behave as if they were being dragged, so to speak, against the stream. They exhibit a peculiar bobbing movement which is distinctly different from Brownian movement. The individual granules tend to arrange themselves on a linear series and do not change their relative positions despite the irregularity of their movement. Later, when contraction nears completion and the pigment reaches the stouter trunk-like origin of each branch, the linear arrangement of the granules is even more accentuated, but the bobbing movements have practically subsided. These observations appear to reinforce two main points in the sol-gel hypothesis: first that the pigment granules are definitely affixed in the contracting gel framework and consequently tend to display a definite pattern of arrangement; and second, that the contraction of the gel framework generates an outward flow of the hyaline sol, derived partly from the central fund of plasmasol and partly from the interstices of the gel itself.

No evidence can be offered as to the mechanism of relaxation, which redistributes the pigment granules after a contraction has abated, except that the protoplasm of the pigment cells always shows a definite degree of solation when relaxation occurs. Compared to the very firm gelation of the contracted state, this solation may be just as great as the solations which have been demonstrated in such relatively loose gels as the plasmagel of the amoeba. But the whole system of the melanophore is pitched at a higher level of gelation. This is indicated by the great centrifugal force necessary to displace the pigment even in the relaxed cells. In the amoeba, a force of less than 7000 gravity is adequate to displace all granules even when the plasmagel is set to its maximum firmness, but in the melanophore a ten times greater force (70,000) is needed, even when the gel system is at minimum "solidity." Apparently there is a very definite residuum of gel structure in the melanophore protoplasm even under conditions of maximum solation. Consequently it is possible that relaxation results from an unfolding of this persistant gel mesh-work, by a reversal of the same processes which determine its contractile folding. In any event it is plain that redistribution of the pigment does not depend on Brownian movement. In drastically centrifuged melanophores, in which presumably the pigment granules have been torn loose from their connection with the gel structure, more than an hour elapses before the displaced granules reach the periphery of the cleared protoplasm in the body and branches of the pigment cells.

Summary

(1) Increasing hydrostatic pressure progressively inhibits the concentration of melanophore pigment, at least roughly in proportion to the magnitude of the pressure, in the range up to 7000 pounds per square inch. At each higher pressure the capacity to contract is further reduced, not only in the case of pulsating melanophores (Spaeth method), but also in the case of steady contractions induced by various chemical agents.

(2) This action of pressure is entirely independent of the nerve supply of the melanophores, since denervation does not in any way alter the pressure responses of the pigment cells.

(3) Low temperature (6° C.) reinforces the pressure inhibition of contraction, but high temperature (30° C.) has a counteracting effect.

(4) Both the pressure and the temperature effects indicate that contraction depends upon the capacity of the protoplasm of the pigment cells to undergo gelation; whereas expansion involves solation. This hypothesis, which is borne out by a number of microscopic observations, brings melanophore activity into line with several other types of protoplasmic movement.

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